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# Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice

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**In chronic kidney disease, fibroblast dysfunction causes renal fibrosis and renal anemia. Renal fibrosis is mediated by the accumulation of myofibroblasts, whereas renal anemia is mediated by the reduced production of fibroblast-derived erythropoietin, a hormone that stimulates erythropoiesis. Despite their importance in chronic kidney disease, the origin and regulatory mechanism of fibroblasts remain unclear. Here, we have demonstrated that the majority of erythropoietin-producing fibroblasts in the healthy kidney originate from myelin protein zero–Cre (*P0-Cre*) lineage-labeled extrarenal cells, which enter the embryonic kidney at E13.5. In the diseased kidney, *P0-Cre* lineage-labeled fibroblasts, but not fibroblasts derived from injured tubular epithelial cells through epithelial-mesenchymal transition, transdifferentiated into myofibroblasts and predominantly contributed to fibrosis, with concomitant loss of erythropoietin production. We further demonstrated that attenuated erythropoietin production in transdifferentiated myofibroblasts was restored by the administration of neuroprotective agents, such as dexamethasone and neurotrophins. Moreover, the *in vivo* administration of tamoxifen, a selective estrogen receptor modulator, restored attenuated erythropoietin production as well as fibrosis in a mouse model of kidney fibrosis. These findings reveal the pathophysiological roles of *P0-Cre* lineage-labeled fibroblasts in the kidney and clarify the link between renal fibrosis and renal anemia.**

## Introduction

Erythropoietin (EPO) is a hormone that is essential for the production of red blood cells (1). EPO is mainly produced in the kidney after birth, and its production is severely reduced in patients with chronic kidney disease (CKD) (2) with renal anemia. The cloning of the *EPO* gene and the production of recombinant human EPO revolutionized the management of patients with CKD, providing an opportunity for safe long-term anemia correction, which improves cognitive function, quality of life, exercise capacity, and cardiac function (3). Nowadays, an increasing number of patients with CKD receive erythropoiesis-stimulating agents (ESAs), and annual US prescription sales for ESAs reached 10 billion dollars in 2006. In addition to the hematopoietic function of EPO, increasing evidence suggests that EPO plays a role as a tissue-protective factor in various nonhematopoietic tissues (4), raising the possibility that it will be prescribed for various types of tissue injuries in the near future.

Studies using *in situ* hybridization and the transgenic mice approach indicate that EPO is mainly produced by the interstitial

fibroblasts in the deep cortex and the outer medulla in the kidney (5, 6). Although the essential role of EPO has been well recognized, the identity and developmental origin of EPO-producing cells in the kidneys remain unknown. This has hindered both the generation of EPO-producing cells from stem cells as well as the clarification of the molecular mechanisms underlying the defective production of EPO in patients with CKD – EPO-producing cells may lose their ability to produce EPO after injury, or, alternatively, EPO-producing cells may be lost during CKD.

Fibroblasts are interstitial mesenchymes that structurally support epithelia by producing ECM. Recently, the tissue-resident fibroblasts have attracted considerable attention because of their prominent roles in fibrosis, endocrine, organogenesis, and tumorigenesis (7). In chronic kidney injury, sustained inflammation accompanies the proliferation of interstitial fibroblasts and myofibroblasts (8), leading to renal fibrosis, which is the final common pathway for all CKD and eventually leads to renal failure (9). The origin of scar-producing myofibroblasts is of prime importance in understanding the mechanisms of renal fibrosis, which remain controversial. The contribution of bone marrow-derived fibrocytes (10) and the epithelial-mesenchymal transition (EMT) of injured tubular epithelial cells (11) has been proposed in some studies, but other groups have reported conflicting results (12–16).

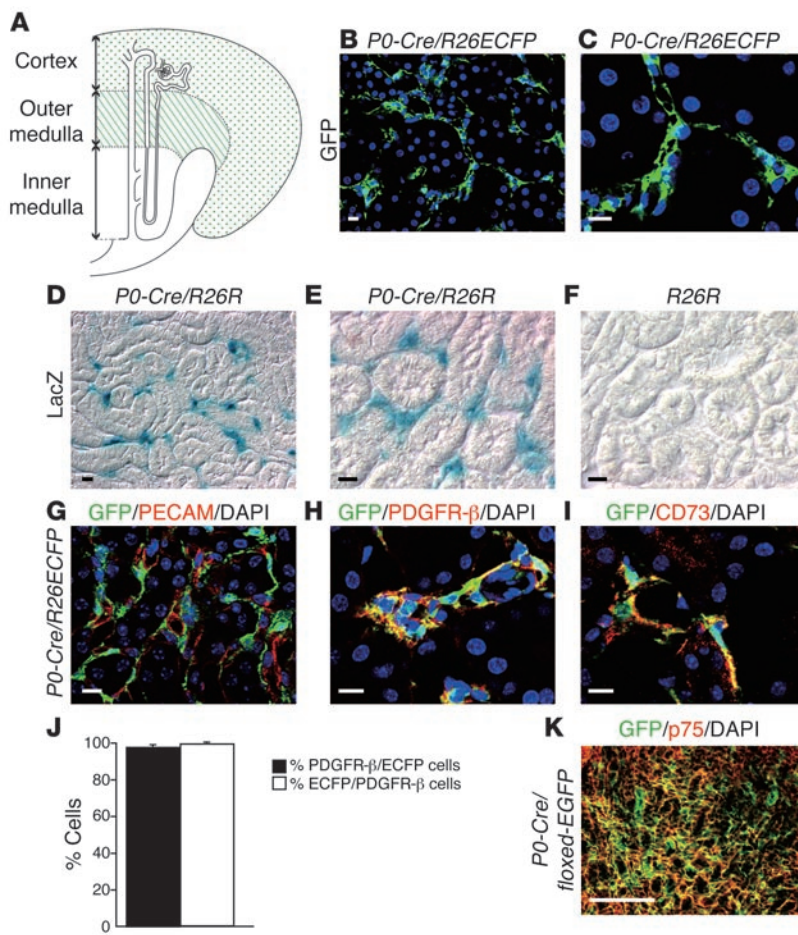
**Authorship note:** Nariaki Asada and Masayuki Takase contributed equally to this work.

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## research article

**Figure 1**

Most of the fibroblasts in the kidney arise from *P0-Cre*-expressing precursors. (A) Schematic drawing of the kidney, showing the localization of *P0-Cre* fate-mapped cells in the cortex and outer medulla. (B and C) ECFP<sup>+</sup> cells were detected in the interstitium of the kidneys of *P0-Cre/R26ECFP* mice. A higher-magnification view is shown in C. (D–F) LacZ staining of *P0-Cre/R26R* kidney. (D and E) Note the similar distribution of LacZ<sup>+</sup> cells in *P0-Cre/R26R* kidneys with ECFP<sup>+</sup> cells in *P0-Cre/R26ECFP* mice. A higher-magnification view is shown in E. (F) No LacZ<sup>+</sup> cells were observed in the kidneys of *R26R* mice without the *P0-Cre* allele. (G–I) Double immunostaining of ECFP<sup>+</sup> cells in the *P0-Cre/R26ECFP* kidney. (G) Most, if not all, of the ECFP<sup>+</sup> cells were negative for PECAM. (H and I) ECFP<sup>+</sup> cells (H) in the kidney were positive for PDGFR-β, the fibroblast marker, whereas ECFP<sup>+</sup> cells (I) in the cortex were also positive for CD73/5'NT, the marker for cortical fibroblasts. (J) Graph illustrating the proportion of PDGFR-β<sup>+</sup> interstitial cells in the cortex and outer medulla coexpressing ECFP (% ECFP/PDGFR-β cells) and the proportion of ECFP<sup>+</sup> interstitial cells coexpressing PDGFR-β (% PDGFR-β/ECFP cells). (K) In neonatal kidneys of *P0-Cre/floxed-EGFP* mice most of the EGFP<sup>+</sup> cells were also positive for p75, a neural crest marker. Scale bars: 10 μm (B–I); 100 μm (K).

Myelin protein zero (P0) is expressed in migrating neural crest cells in the early embryonic stages as well as in Schwann cells, which also originate from neural crest, in later stages (17). In *P0-Cre* mice, the activation of the *P0* promoter induces the Cre-mediated recombination of reporter alleles, permanently tagging the cells arising from *P0-Cre*-expressing precursors with the expression of indicator genes.

Here, we demonstrate the occurrence of *P0-Cre* lineage-labeled fibroblasts in the kidney interstitium and their 2 different characteristics — they produce EPO, which possesses hematopoietic and tissue-protective functions in healthy kidney, and they transdifferentiate into scar-producing myofibroblasts and lose their EPO-producing activity after kidney injury, leading to renal fibrosis and renal anemia. We further demonstrate that the attenuated EPO production in scar-producing myofibroblasts can be restored through the administration of neuroprotective reagents, such as low-dose dexamethasone and neurotrophins. A similar effect was also obtained by the administration of a renoprotective reagent HGF. Moreover, the in vivo administration of tamoxifen, a selective estrogen receptor modulator (SERM), restored attenuated EPO production as well as fibrosis in a mouse model of kidney fibrosis.

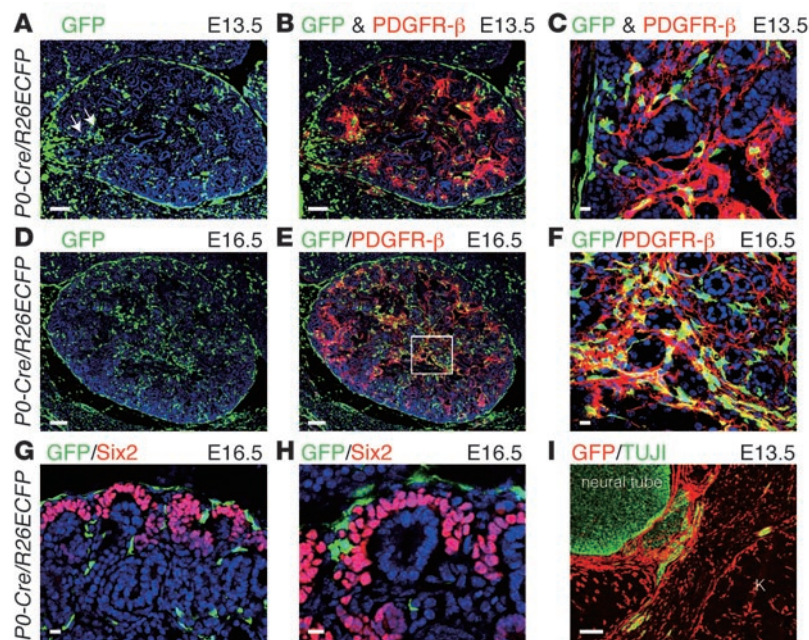
These findings provide insights that we believe to be novel into the mechanisms of renal fibrosis and renal anemia in CKD and provide clues that assist in the development of therapeutic approaches to treat these disorders.

## Results

*Most fibroblasts in the kidney arise from P0-Cre-expressing precursors.* *P0-Cre* mice were mated to 4 indicator mouse lines: *floxed EGFP* (18), *R26R* (19), *R26ECFP* (20), and *R26tdRFP* mice (21). Immunostaining of the kidneys of *P0-Cre/R26ECFP* mice using anti-GFP antibody revealed numerous enhanced cyan fluorescent protein-positive (ECFP<sup>+</sup>) cells in the cortex and outer medulla (Figure 1, A–C). The kidneys of *R26ECFP* mice without the *P0-Cre* allele contained no ECFP<sup>+</sup> cells, confirming the specificity of staining (data not shown). LacZ staining in *P0-Cre/R26R* kidney also revealed the same results (Figure 1, D–F). These *P0-Cre* fate-mapped cells are located in the interstitium, the space between the tubules and nutritive peritubular capillaries, exhibiting a unique stellar shape with numerous projections.

The interstitium of the healthy adult kidney is composed of heterogeneous cells, including macrophages, dendritic cells, and peritubular capillaries (9). Most, if not all, *P0-Cre* fate-mapped cells in the kidney did not express the macrophage marker CD11b (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI57301DS1), F4/80 (Supplemental Figure 1B), the dendritic cell marker major histocompatibility complex class II (Supplemental Figure 1C and Supplemental Figure 7), or the common leukocyte antigen CD45 (Supplemental Figure 7). Although a previous report indicated that endothelial cells in certain tissues are of neural crest origin (22), ECFP<sup>+</sup> cells in the kidney of *P0-Cre/R26ECFP* mice did not express PECAM/



**Figure 2**

*P0-Cre* lineage-labeled cells in the developing kidney. (A–C) ECFP<sup>+</sup> cells were first observed in the kidneys of *P0-Cre/R26ECFP* mice at E13.5. ECFP<sup>+</sup> cells were predominantly located along the outer capsule and the ureter (arrows) of the kidney. (D–F) The number of ECFP<sup>+</sup> cells in the kidneys of *P0-Cre/R26ECFP* mice increased at E16.5, and (F) the cells began to express PDGFR-β. The boxed region in E is shown at higher magnification in F. (G and H) ECFP<sup>+</sup> cells populating the cortex appeared to surround the territory of Six2<sup>+</sup> renal progenitor cells. (I) ECFP<sup>+</sup> cells in the kidney were negative for class III β-tubulin. K, kidney. Scale bars: 100 μm (A, B, D, E, and I); 10 μm (C and F–H).

CD31, a marker for endothelial cells (Figure 1G and Supplemental Figure 7). Very few ECFP<sup>+</sup> cells expressed tyrosine hydroxylase, a marker for sympathetic nerve fibers, whereas most ECFP<sup>+</sup> cells did not (Supplemental Figure 2). Instead, ECFP<sup>+</sup> cells expressed PDGFR-β, a marker for interstitial fibroblasts in the kidney (refs. 9, 23, and Figure 1H). ECFP<sup>+</sup> cells in the cortex expressed CD73/ecto-5'-nucleotidase (CD73/5'NT), a marker for cortical fibroblasts (ref. 9 and Figure 1I). More than 98% of ECFP<sup>+</sup> interstitial cells expressed PDGFR-β, whereas more than 99% of PDGFR-β<sup>+</sup> cells in the interstitium of the cortex and outer medulla expressed ECFP (Figure 1J). Furthermore, EGFP<sup>+</sup> cells in the neonatal kidneys of *P0-Cre/floxed-EGFP* mice expressed p75, a marker for the neural crest (ref. 24 and Figure 1K). These results indicate that most, if not all, of the kidney fibroblasts in the cortex and outer medulla are fate-mapped with *P0-Cre*.

*P0-Cre* lineage-labeled cells enter the embryonic kidney during nephrogenesis. To investigate the route and stage of migration of *P0-Cre* lineage-labeled cells into the kidney, we analyzed *P0-Cre/R26ECFP* embryos serially from E11.5, when nephrogenesis is initiated, to the neonatal stage. ECFP<sup>+</sup> cells were first detected from E13.5 onward in metanephric kidneys. These cells were predominantly located along the outer capsule and the ureter of the kidney (Figure 2, A–C), which is possibly indicative of their routes of migration. The number of ECFP<sup>+</sup> cells in the metanephric kidney dramatically increased in later stages of development, and the ECFP<sup>+</sup> cells began to express PDGFR-β (Figure 2, D–F).

These ECFP<sup>+</sup> cells populating the cortex appeared to avoid the territory of the Six2<sup>+</sup> renal progenitor cells, which give rise to all nephron segments in the kidney (ref. 25 and Figure 2, G and H). Furthermore, these ECFP<sup>+</sup> cells did not express class III β-tubulin, a neuron-specific marker (Figure 2I). Importantly, immunostaining of the *P0-Cre* embryo failed to identify any Cre<sup>+</sup> cells in the kidneys throughout development (Supplemental Figure 3, C–E), whereas it detected the expression of Cre in the migrating neural crest and the dorsal root ganglion (Supplemental Figure 3, A and B). Immunostaining of P0 protein also failed to detect any P0

protein in the kidneys throughout development (Supplemental Figure 4, A–C). We further demonstrated that the expression of *P0* and *Cre* mRNA was almost undetectable in embryonic kidneys (Supplemental Figure 8), whereas the number of *P0-Cre*-labeled cells increased substantially during development. Thus, it is highly unlikely that the ectopic expression of Cre in the kidney accounts for ECFP<sup>+</sup> cells in the kidney.

*P0-Cre* lineage-labeled fibroblasts produce EPO. Renal EPO-producing cells are thought to be peritubular interstitial cells that exhibit a stellate shape with projections and express neuronal markers (6). Given these features of EPO-producing cells, we examined whether EPO-producing cells arise from *P0-Cre*-expressing precursors. We used *Epo-GFP* mice, a BAC transgenic mouse line that expresses GFP as a reporter under the control of a 180-kb mouse *Epo* gene locus, and identified the EPO-producing population in the kidney (6).

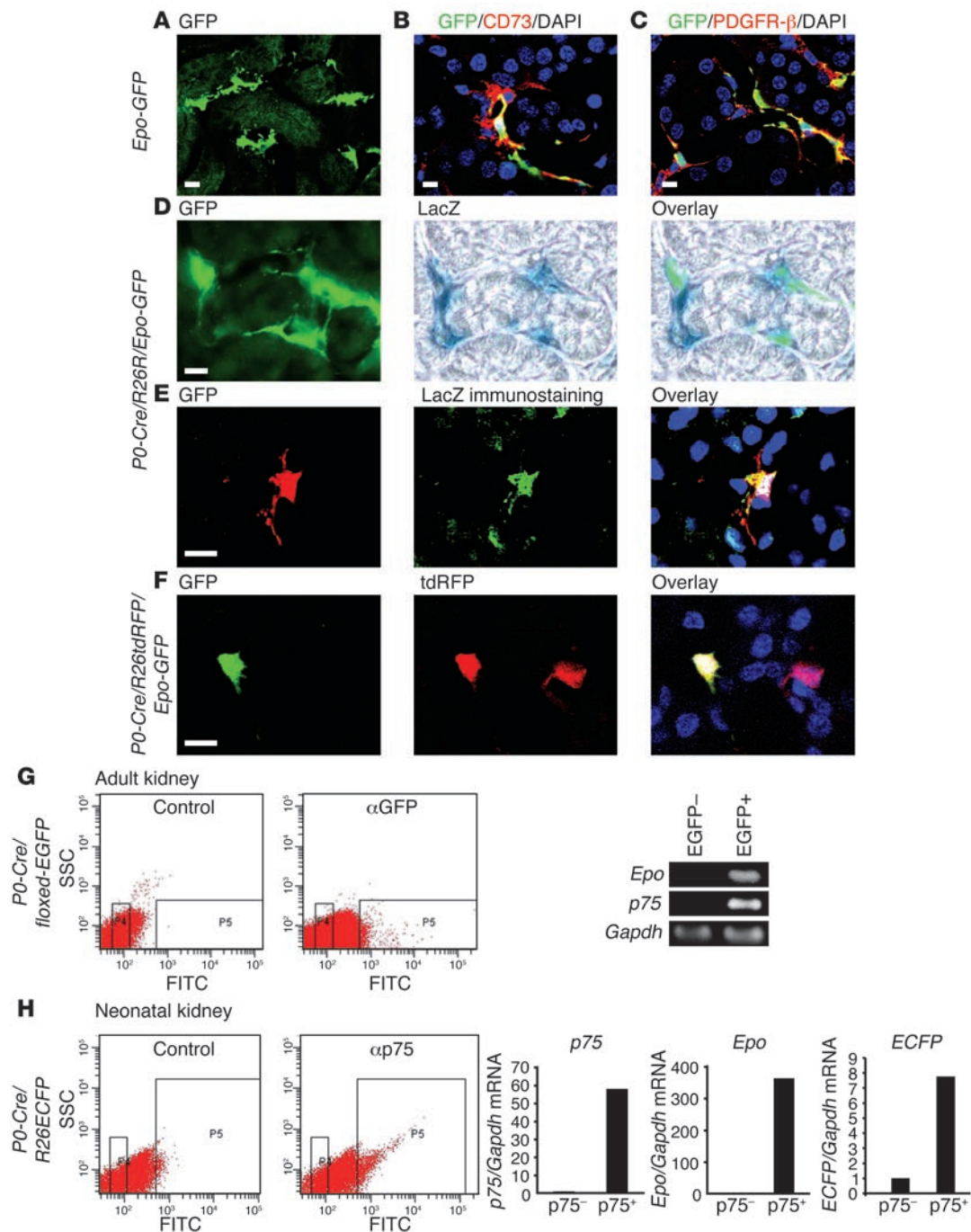
GFP expression in the kidney of *Epo-GFP* mice was detected in the interstitium and was colocalized with the fibroblast markers CD73/5'NT and PDGFR-β (Figure 3, A–C). Analysis of the kidneys of *P0-Cre/R26R/Epo-GFP* mice revealed that most of the GFP<sup>+</sup> cells were positive for LacZ (Figure 3, D and E), supporting the idea that EPO-producing cells originate from the *P0-Cre*-expressing precursors. We further analyzed the kidneys of *P0-Cre/R26tdRFP/Epo-GFP* mice and found that more than 75% of GFP<sup>+</sup> cells were also positive for tandem-dimer red fluorescent protein (tdRFP) (Figure 3F).

EPO expression in *P0-Cre* fate-mapped cells was further examined using EGFP<sup>+</sup> and EGFP<sup>−</sup> cells FACS-purified from kidneys of adult *P0-Cre/floxed-EGFP* mice. PCR analysis of these populations revealed the expression of EPO and p75 in EGFP<sup>+</sup> cells but not in the EGFP<sup>−</sup> population (Figure 3G). Sorting p75<sup>+</sup> cells in the kidneys of *P0-Cre/R26ECFP* mice at 2 weeks of age also revealed higher expression of p75, EPO, and ECFP in p75<sup>+</sup> cells than in p75<sup>−</sup> cells (Figure 3H).

*P0-Cre* lineage-labeled fibroblasts transdifferentiate into myofibroblasts and contribute to fibrosis. To investigate the response of *P0-Cre* lineage-labeled fibroblasts to injury, we performed unilateral ureteral obstruction (UUO), a well-established model for renal fibrosis

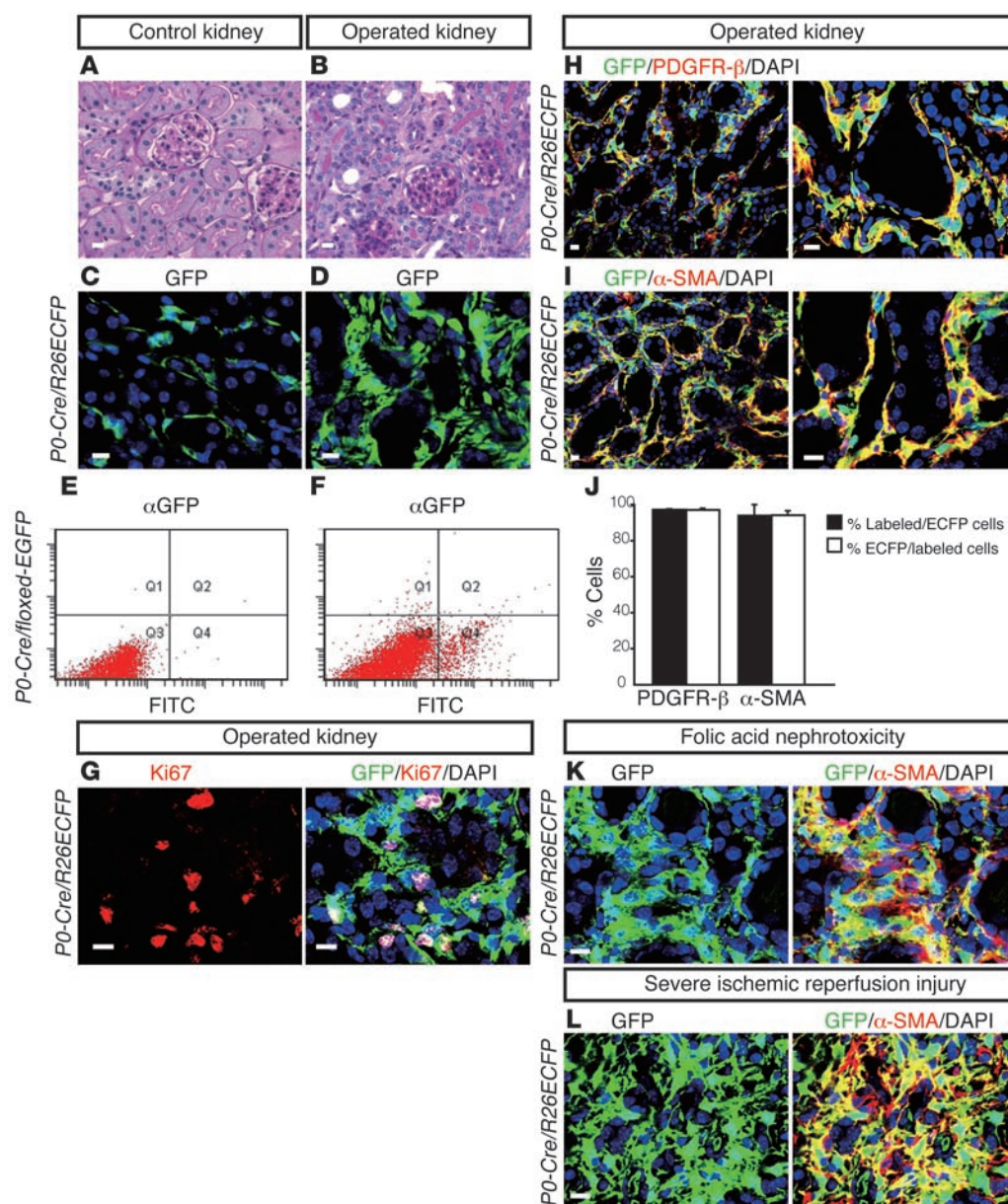


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**Figure 3**

*P0-Cre* lineage-labeled fibroblasts produce EPO. (**A–C**) Stellate-shaped GFP<sup>+</sup> cells with projections were detected (**A**) in the interstitium of the kidneys of *Epo-GFP* mice and were positive for (**B**) CD73 and (**C**) PDGFR- $\beta$ . (**D** and **E**) Analysis of the kidney of *P0-Cre/R26R/Epo-GFP* mice revealed that GFP<sup>+</sup> cells were also positive for LacZ. (**F**) Analysis of the kidney of *P0-Cre/R26tdRFP/Epo-GFP* mice revealed that most GFP<sup>+</sup> cells were also positive for tdRFP. Scale bars: 10  $\mu$ m. (**G**) Sorting EGFP<sup>+</sup> cells (P5) out of EGFP<sup>-</sup> cells (P4) from the kidneys of adult *P0-Cre/floxed-EGFP* mice. RT-PCR analysis of these populations revealed high expression of *Epo* and *p75* in EGFP<sup>+</sup> cells but not in EGFP<sup>-</sup> cells. Cells were stained either with or without anti-GFP antibody. (**H**) Sorting p75<sup>+</sup> cells (P5) out of p75<sup>-</sup> cells (P4) from the kidneys of *P0-Cre/R26ECFP* mice at 2 weeks of age. Cells were stained either with or without anti-p75 antibody. Quantitative PCR analysis demonstrated that the expression of *p75*, *Epo*, and *ECFP* was higher in p75<sup>+</sup> cells. Expression of *p75* was normalized to that of *Gapdh* and expressed relative to that in p75<sup>-</sup> cells.





#### Figure 4

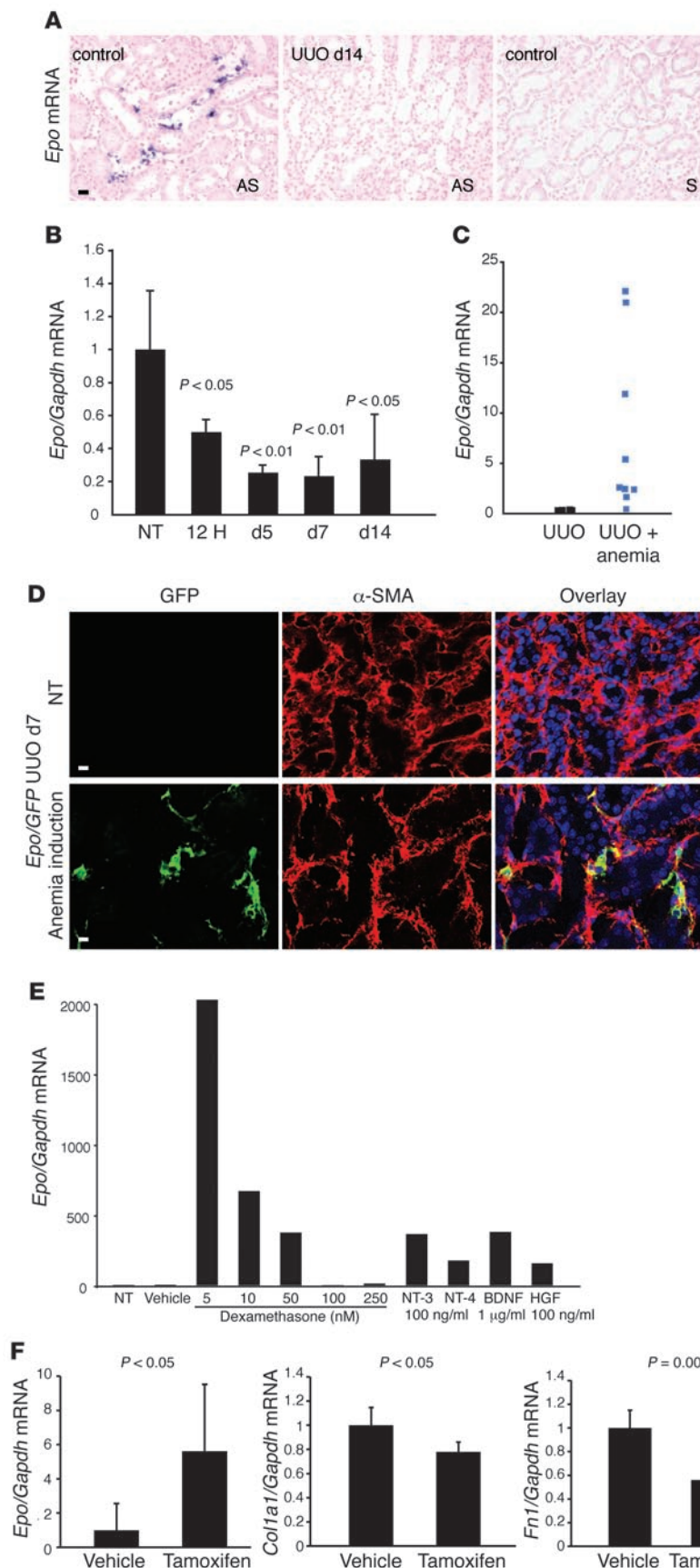
*P0-Cre* lineage-labeled fibroblasts transdifferentiate into myofibroblasts and contribute to fibrosis. (A and B) Five days after obstruction of the ureter, (B) interstitial fibrosis was prominent in the operated kidney (A) compared with that in the control kidney. (C and D) The number of ECFP<sup>+</sup> cells was markedly increased in the operated kidneys of *P0-Cre/R26ECFP* mice compared with that in the control kidney. (E and F) FACS analysis also confirmed the increase in the number of EGFP<sup>+</sup> cells in the operated kidneys of *P0-Cre/floxed-EGFP* mice. Cells were stained with anti-GFP antibody. (G) ECFP/Ki67 double-positive cells were abundant in the operated kidneys of *P0-Cre/R26ECFP* mice. (H and I) Most ECFP<sup>+</sup> cells were also positive for (H) PDGFR- $\beta$  and (I)  $\alpha$ -SMA. (J) Graph illustrating the proportion of PDGFR- $\beta$ <sup>+</sup> or  $\alpha$ -SMA<sup>+</sup> interstitial cells coexpressing ECFP (% labeled/ECFP cells) and the proportion of interstitial ECFP<sup>+</sup> cells coexpressing either PDGFR- $\beta$  or  $\alpha$ -SMA (% ECFP/labeled cells). (K and L) The number of ECFP<sup>+</sup> cells was also markedly increased in the kidneys of *P0-Cre/R26ECFP* mice after (K) folic acid nephrotoxicity and (L) severe ischemic reperfusion injury. Most ECFP<sup>+</sup> cells were also positive for  $\alpha$ -SMA. Scale bars: 10  $\mu$ m.

(26), using *P0-Cre/R26ECFP* mice. Five days after UUO, the number of ECFP<sup>+</sup> cells had markedly increased in the interstitium of the operated kidney, in parallel with the progression of interstitial fibrosis (Figure 4, A–D). FACS analysis also revealed a substantial increase in the number of EGFP<sup>+</sup> cells in the operated kidney in *P0-Cre/floxed-EGFP* mice (Figure 4, E and F). It is unlikely that the increase in ECFP<sup>+</sup> or EGFP<sup>+</sup> cells is due to the ectopic expression

of Cre or P0 protein in the kidney, because neither Cre protein nor P0 protein was detectable in the operated kidney (Supplemental Figure 3F and Supplemental Figure 4D). We further demonstrated that the expression of *P0* and *Cre* mRNA was almost undetectable in fibrotic kidneys (Supplemental Figure 8), whereas the number of *P0-Cre*-labeled cells increased substantially during kidney fibrosis. Immunostaining of the operated kidney revealed the presence



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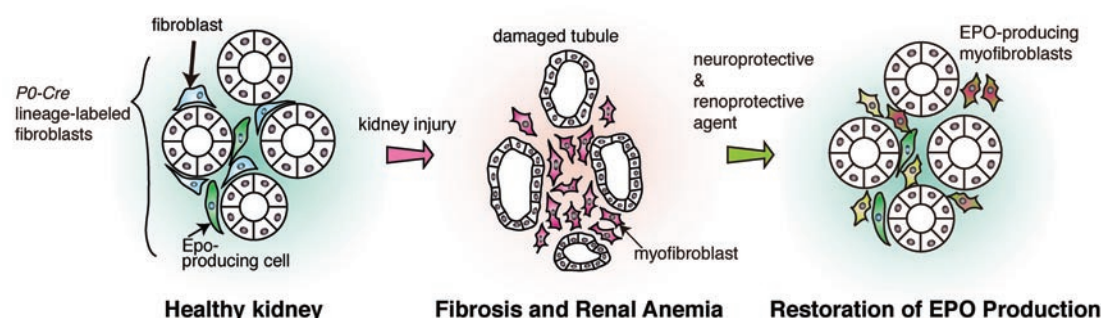
**Figure 5**

EPO-producing fibroblasts also transform into myofibroblasts, hindering EPO production. (A) In situ hybridization demonstrated that *Epo* mRNA detected in the control kidney was almost undetectable in the operated kidneys at day 14 of UUO. Hybridization with a sense probe did not elicit any signals in the control kidney. AS, antisense; S, sense. (B) Expression of *Epo* mRNA in the operated kidney decreased significantly, as early as 12 hours after ligation of the ureter ( $n = 5$ /group, except for the group sacrificed at day 5 [d5], in which  $n = 4$ ). NT, nontreated. (C) The suppression of *Epo* mRNA in the operated kidneys was reversed after the induction of anemia. Each dot represents renal expression of *Epo* in an individual mouse ( $n = 5$  in UUO group;  $n = 9$  in UUO plus anemia group). (D) Undetectable GFP<sup>+</sup> cells in the operated kidneys of *Epo*-GFP mice became detectable after the induction of anemia and colocalized with  $\alpha$ -SMA. (E) The expression of *Epo* in cultured myofibroblasts was augmented by the administration of low-dose dexamethasone, neurotrophins, and HGF. Vehicle used was 0.05% ethanol for the control of dexamethasone. Data are representative of results of 5 independent experiments. (F) Expression of *Epo* mRNA in UUO kidneys was significantly restored, whereas the expression of *Col1a1* and fibronectin (*Fn1*) mRNA was decreased by tamoxifen treatment ( $n = 5$ ). Vehicle used was 100  $\mu$ l of 10% ethanol in sunflower oil. Expression of *Epo*, *Col1a1*, and *Fn1* was normalized to that of *Gapdh* and expressed relative to that in (B and E) the nontreated group or (F) vehicle-treated group. Scale bars: 10  $\mu$ m.

of ECFP/Ki67 double-positive cells, indicating that the increase in the number of ECFP<sup>+</sup> cells in the operated kidney is at least partially attributable to the proliferation of ECFP<sup>+</sup> cells (Figure 4G).

Another possibility is that macrophages and dendritic cells infiltrated the operated kidneys in response to inflammation. This would account for the increase in the number of ECFP<sup>+</sup> cells in the operated kidneys. However, ECFP<sup>+</sup> cells did not overlap with the inflammatory cells, which infiltrated the operated kidneys in response to inflammation (Supplemental Figure 1, D–F). Instead, these ECFP<sup>+</sup> cells were almost exclusively positive for PDGFR- $\beta$  (Figure 4H), a marker for fibroblasts, and  $\alpha$ -SMA (Figure 4I), a marker for myofibroblasts. Quantitative analysis demonstrated that 97% of PDGFR- $\beta$ <sup>+</sup> interstitial cells as well as 94% of  $\alpha$ -SMA<sup>+</sup> myofibroblasts were positive for ECFP, whereas 97% and 93% of ECFP<sup>+</sup> cells were positive for PDGFR- $\beta$  and  $\alpha$ -SMA, respectively (Figure 4J). Similar results were



**Figure 6**

Hypothetical model for 2 different characteristics of *P0-Cre* lineage-labeled fibroblasts in the kidney, with a possible therapeutic implication for treatment of renal anemia. *P0-Cre* lineage-labeled fibroblasts in the kidney produce EPO, which possesses hematopoietic and tissue-protective functions in healthy kidneys. In contrast, they transdifferentiate into scar-producing myofibroblasts and lose EPO-producing activity after kidney injury, leading to renal fibrosis and renal anemia. We further demonstrated that the administration of various neuroprotective and renoprotective reagents restores the EPO-producing ability in fibrotic kidneys.

obtained in other models of renal fibrosis — folic acid nephropathy (Figure 4K) and severe ischemic reperfusion injury (Figure 4L). These results indicate that *P0-Cre* lineage-labeled fibroblasts are the main source of scar-producing myofibroblasts, contributing to fibrosis after kidney injury.

*EPO-producing fibroblasts also transform into myofibroblasts at the cost of EPO production.* Regardless of the cause of the damage, diseased kidneys invariably suffer from fibrosis with reduced EPO production. The mechanism underlying this defective production of EPO in the diseased kidney remains unknown (27). In situ hybridization detected *Epo* mRNA in the interstitium of the control kidney (Figure 5A, left) but not in the operated kidney at day 14 of UUO (Figure 5A, middle). Suppression of *Epo* mRNA in the operated kidney was evident as early as 12 hours after ligation of the ureter (Figure 5B). Interestingly, the attenuated production of *Epo* mRNA in the fibrotic kidney was restored by the induction of anemia (Figure 5C and Supplemental Figure 10). Similarly, GFP<sup>+</sup> cells were almost undetectable in the fibrotic kidneys of *Epo-GFP* mice, whereas many GFP<sup>+</sup> cells emerged in the fibrotic kidneys after the induction of anemia (Figure 5D). These GFP<sup>+</sup> cells in the fibrotic kidneys were positive for  $\alpha$ -SMA (Figure 5D), whereas GFP<sup>+</sup> cells in the healthy kidneys were negative for  $\alpha$ -SMA (Supplemental Figure 5). These results indicate that transdifferentiated myofibroblasts still possess the capacity to produce EPO after the induction of anemia.

Taken together, the results indicate that the cause of renal anemia is not the loss of EPO-producing cells but is at least partially due to the transdifferentiation of EPO-producing cells into scar-producing myofibroblasts that express a smaller amount of EPO (Figure 6).

These findings led us to seek the possible stimuli to restore the EPO-producing ability in myofibroblasts. Because EPO-producing cells in the kidney possess both renal and neural characteristics, we administered renoprotective and neuroprotective agents to cultured primary myofibroblasts obtained from fibrotic kidneys to determine whether or not the EPO-producing ability could be restored. Among the agents that we tested, we found that the administration of neuroprotective agents, such as low-dose dexamethasone, neurotrophins (neurotrophin-3 [NT-3], NT-4, and brain-derived neurotrophic factor [BDNF]), and the renoprotective agent, HGF, increased EPO production in myofibroblasts (Figure 5E and Supplemental Figure 11). We sought

other possible reagents to alleviate the loss of EPO in fibrotic kidney in vivo and found that the administration of tamoxifen, a SERM, restored the attenuated expression of EPO in fibrotic kidney (Figure 5F). SERMs activate the estrogen receptors and function as estrogen agonists in some tissues, such as bone and kidneys, and as estrogen antagonists in other tissues, such as breast. The administration of tamoxifen also restored fibrosis significantly (Figure 5F). These results indicate that the attenuated production of EPO in scar-producing myofibroblasts can be restored (Figure 6).

## Discussion

In the present study, we demonstrated that EPO-producing cells in healthy kidney and scar-producing myofibroblasts during fibrosis originate from the same *P0-Cre* lineage-labeled extrarenal cells, which enter the embryonic kidney at E13.5 to become renal fibroblasts and transit from one another depending on the condition of the kidney. A greater understanding of the mechanisms that control the balance between these 2 functions will lead to better treatment for patients with CKD. More importantly, the reversibility of EPO production in the fibrotic kidney (Figure 5, C–F) raised the possibility of a therapeutic approach toward renal anemia (Figure 6).

Neurotrophins are reported to promote neuronal growth and survival in many neuronal populations through the activation of Trk receptor tyrosine kinases (28–30). Furthermore, NT-3 rescues neuronal precursors from apoptosis and promotes neuronal differentiation in embryonic kidney explants (31). Given the origin of EPO-producing cells from *P0-Cre*-expressing precursors, it is plausible that neurotrophins restore EPO-producing ability in transdifferentiated myofibroblasts. Recently, pharmaceutical strategies to stimulate neurotrophin signaling established proof of principle of these as effective therapeutics for Alzheimer disease (30). Among these strategies, small molecule activators of neurotrophin are appealing and should be tested in the model of renal anemia in the future.

Glucocorticoid exhibits both protective and destructive effects in the nervous system. In excess, glucocorticoid produces neuronal death, whereas low-dose glucocorticoid is neuroprotective. Recently, it has been demonstrated that glucocorticoid provides a neuroprotective effect through the activation of neurotrophin signaling





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(32), indicating that the beneficial effect of low-dose dexamethasone in the restoration of the EPO-producing ability might be exerted through neurotrophin functions as well.

On the other hand, HGF has been reported to promote the regeneration of damaged cells after kidney injury (33). The regenerative capacity of HGF may be effective in the restoration of EPO-producing ability in myofibroblasts.

Recently, Bernhardt et al. demonstrated that the pharmacological manipulation of the HIF system increases EPO production in patients with end-stage renal disease. They suggested that the disturbed oxygen sensing of EPO-producing cells causes renal anemia (34). However, the activation of HIF has multiple downstream effects, and intermittent HIF activation over a prolonged period of time may lead to profound changes in cellular metabolism, growth, and differentiation (35).

The results of the present study prompt the proposal of another possible therapeutic approach to treat renal anemia by protecting damaged EPO-producing cells. The administration of neurotrophins, low-dose dexamethasone, and HGF as well as the reagents activating these pathways may be a promising therapeutic approach either alone or in combination with other approaches. In addition, SERMs, including tamoxifen, are promising candidate drugs to treat renal anemia as well as kidney fibrosis. Recently, raloxifene, another SERM, has been reported to be renoprotective in postmenopausal women with osteoporosis (36). Clinical trials of SERMs in patients with CKD with renal anemia and further clarification of the mechanism of action of SERMs are needed.

Although we demonstrated that almost all cortical fibroblasts in the kidney arise from *P0-Cre*-expressing precursors (Figure 1J), less than 20% of cortical fibroblasts produce EPO, even at the maximum induction of EPO under an anemic or hypoxic condition (27). Whether or not a certain subpopulation of *P0-Cre* lineage-labeled fibroblasts is designated to possess EPO-producing ability, or all *P0-Cre* lineage-labeled fibroblasts are born with the ability but some environmental factors determine which cells produce EPO, remains to be clarified. Although the lack of specific cell surface markers of EPO-producing cells has hindered further analysis, we demonstrated that p75 is an effective surface marker in the purification of EPO-producing cells in neonatal kidney (Figure 3H). In the adult kidney, however, p75 was not an effective marker because the expression of p75 protein in the kidney was downregulated (data not shown), whereas its mRNA was still detected by reverse-transcription PCR (RT-PCR) in EPO-producing cells (Figure 3G). A genome-wide analysis of EPO-producing cells purified from neonatal kidneys may enable the identification of suitable surface markers for the purification of the cells in adult kidneys.

We also demonstrated that more than 94% of myofibroblasts arise from *P0-Cre* lineage-labeled fibroblasts, providing what we believe to be new insights into the long-lasting controversy regarding the source of scar-producing myofibroblasts in the kidney. Recently, EMT has become widely accepted as a mechanism by which injured tubular cells transform into scar-producing cells and contribute to fibrosis during kidney injury (11). However, a number of conflicting data about the existence of EMT *in vivo* have been published (8, 37–39). Our data support the idea that myofibroblasts emerge from the proliferation and transdifferentiation of fibroblasts in the kidney (8), but it is not likely that they emerge from EMT of injured tubules. Recently, Humphreys et al. proposed that FoxD1<sup>+</sup> mesenchymal cells in the developing kidneys differentiate into myofibroblasts in

fibrosis (37), although the labeling efficiency of myofibroblasts by *FoxD1*-inducible *Cre* transgenic mice was 20%, due to the toxicity of tamoxifen in embryogenesis. *P0-Cre* fate-mapped cells populate around Six2<sup>+</sup> progenitor cells in the developing kidney (Figure 2, G and H), and some of them express FoxD1 (Supplemental Figure 9). From this result, we speculate that the *P0-Cre*-labeled population and the *FoxD1-Cre*-labeled population are overlapping.

Recent reports indicate that neural crest cells contribute to bone marrow cells (22) and mesenchymal stem cells (40), in addition to the classical neural crest derivatives, such as peripheral nervous system, melanocytes, and adrenal medulla (24). The previous chick-quail xenotransplantation experiment (41, 42) as well as the dye injection into the dorsal neural tube (43) indicate that neural crest-derived cells contribute to the developing kidneys (44). This observation is also supported by the results in this manuscript, showing that most fibroblasts in the kidney arose from *P0-Cre*-expressing precursors (Figure 1, H–J) and that neonatal kidney fibroblasts expressed neural crest marker p75 (Figure 1K). On the contrary, a previous lineage-tracing study using *Wnt1-Cre* mice (45), another tool to trace the fate of neural crest derivatives, does not reveal a pattern overlapping with that of *P0-Cre* mice in the kidney. One possible explanation for the discordant results might be a difference in the efficiency and timing of labeling neural crest-derived cells in these *Cre* drivers. For instance, in *Wnt1-Cre* mice, *Cre* is predominantly expressed in the dorsal neural tube from which neural crest cells delaminate, whereas in *P0-Cre* mice, *Cre* expression is sustained in migrating neural crest cells. Although there is a possibility that the *P0-Cre* driver is ectopically expressed in other precursor tissues, *P0-Cre*-driven lineage labeling has to occur outside of the embryonic kidney, since we did not detect *Cre* expression within the developing kidney (Supplemental Figures 3 and 8).

Although further study is needed before one can definitely conclude that *P0-Cre* lineage-labeled cells are of neural crest origin, the hypothesis that EPO-producing cells derive from neural crest cells might open a new avenue of research toward the *in vitro* generation of EPO-producing cells from patient-derived induced pluripotent stem (iPS) cells. This is promising because an efficient method for converting iPS cells to neural crest cells has already been established in a recent study (46).

## Methods

**Animals.** Transgenic mice expressing *Cre* under the control of the *P0* promoter (*P0-Cre* mice) (17) were mated with *floxed-EGFP* mice (*CAG-CAT<sup>loxP</sup>/loxP-EGFP* mice; ref. 18) (a gift from J. Miyazaki of Osaka University, Osaka, Japan), *R26R* mice (19), *R26ECFP* mice (20) (a gift from F. Costantini of Columbia University, New York, New York, USA), and *R26tdRFP* mice (21) to obtain double-transgenic mice. *Epo-GFP* mice (6) were described previously. Adult wild-type mice were purchased from Japan SLC. Anemia was induced by bleeding (6) as well as by the administration of phenylhydrazine as described previously (47). The GFP signal in *Epo-GFP* mice was observed after the induction of anemia. All animal studies were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (US NIH).

**Kidney disease models.** UUO and folic acid nephrotoxicity was induced as described previously (48, 49). Severe ischemic reperfusion injury was induced by 75-minute clamp of the unilateral renal pedicles: the kidneys were harvested 3 weeks after the operation. Tamoxifen (5 mg/20 g BW) was orally administered everyday from 5 days before UUO to the day of operation and every 2 days after the operation.



**Renal histopathology.** Immunostaining was performed as described previously (50) using the following primary antibodies: anti-GFP (Molecular Probes), p75 (Advanced Targeting Systems), PDGFR- $\beta$  (eBioscience), Six2 (Proteintech Group),  $\alpha$ -SMA (Sigma-Aldrich), TUJ1-FITC (Covance), TH (Chemicon), Cre (Novagen), P0 (Novus Biologicals), LacZ (Cappel), RFP (Abcam), FoxD1 (a gift from A.P. McMahon and J.W. Mugford of Harvard University, Cambridge, Massachusetts, USA) (51), and CD11b, CD11c, CD31, CD73 (all from Pharmingen). EGFP and ECFP were visualized by immunostaining with anti-GFP antibody. tdRFP was visualized by immunostaining with anti-RFP antibody.

**Flow cytometric analysis.** Flow cytometric analysis was performed using BD FACSARIA. Mice were anesthetized and then perfused with normal saline. Kidneys were harvested, and the tissue was minced with a razor blade and digested by 1 mg/ml collagenase A and 0.1 mg/ml DNase type I as previously described (52). The cell suspensions were filtered through a cell strainer to remove debris and were stained with antibodies against GFP (Molecular Probes) and p75 (Advanced Targeting Systems). The cells were fixed in 4% PFA for GFP staining but not for p75 staining.

**Myofibroblast culture.** UUO kidneys were harvested 14 days after the operation in the same manner used in the preparation of cell suspension for FACS analysis. Cells from 1 UUO kidney were seeded onto a Primaria 6-well dish (BD Bioscience), washed twice, stimulated with various agents 24 hours later, and incubated 48 hours after the stimulation in a hypoxic chamber (1% O<sub>2</sub>). We confirmed that most, if not all,  $\alpha$ -SMA<sup>+</sup> cultured myofibroblasts from *PO-Cre/R26ECFP* mice were positive for ECFP (Supplemental Figure 6). We also confirmed the presence of EPO protein (0.2–0.75 pg/ml) in the culture supernatant of the cells using ELISA (Mouse/Rat EPO Quantikine ELISA system, R&D Systems).

**Statistics.** All assays were performed at least 5 times. Data are presented as the mean  $\pm$  SD. Statistical significance was assessed by Student's *t* test for 2 group comparisons. *P* < 0.05 is considered significant.

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